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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Only a few epidemiological studies have shown a slight increased risk of breast cancer in cigarette smokers. To evaluate DNA damage due to smoking we studied 4-aminobiphenyl(4-ABP)-DNA adduct levels in breast tumor tissue in a population-based study of 150 cases from New Jersey. All subjects were under the age of 45 with either in situ or invasive breast cancer. Adducts in breast tumor sections of paraffin blocks were measured using an immunohistochemical peroxidase assay. The difference in relative staining intensity for 4-ABP-DNA adducts was not significantly different between smokers, ex-smokers, and non-smokers. Adduct levels was not significantly elevated in smokers with higher compared to lower exposure in relation to age at initiation or pack-years. We then genotyped for enzymes that metabolize 4-ABP; cytochrome P4501A2 (CYP1A2), two N-acetyltransferases, NAT1 and NAT2, and glutathione-S-transferase(GST). None of these genes, independently or in combination, had a significant association with adduct levels. The p53 mutation spectrum is a useful reporter of past genotoxic exposure. We are currently identifying the mutations for exons 5-8 in the p53 gene with the innovative DHPLC assay. We will correlate the mutation data with smoking status and adduct levels to determine if smoking exposure is a risk for breast cancer.

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INTRODUCTION

Only a few epidemiological studies have shown a slight increased risk of breast cancer in cigarette smokers. The risk is only seen when variables of smoking status or genotypic variance in metabolizing enzymes are considered. To evaluate smoking exposure we studied 4-aminobiphenyl(4-ABP)-DNA adduct levels in breast tissue in a population-based study of 150 cases from New Jersey. All subjects were under the age of 45 with either *in situ* or invasive breast cancer. Adducts in breast tumor cells and normal adjacent cells in archived breast tumor sections of paraffin blocks were measured using an immunohistochemical peroxidase assay. 4-ABP is not carcinogenic in the parent form but requires metabolic activation to reactive electrophiles in order to produce its carcinogenic effects. The enzymes that have been found to metabolize 4-ABP are cytochrome P4501A2 (CYP1A2), two N-acetyltransferases, NAT1 and NAT2, and glutathione-S-transferase(GST). The role single nucleotide polymorphsims in the carcinogen metabolizing enzymes, CYP1A2, NAT2, NAT1 and GSTP1 have in predicting 4-ABP-DNA adduct levels were determined. Analysis of the single nucleotide polymorphisms were carried out by polymerase-chain reaction-restriction fragment length polymorphism(PCR-RFLP) assay. Mutations found in the tumor suppressor gene p53 provide important clues to cancer etiology. In breast cancer the mutational spectra of p53 suggests the involvement of carcinogen-DNA adducts. We set out to determine the mutations of p53 gene with the conventional single strand conformation polymorphism(SSCP) method as well as the latest Affymetrix GeneChip assay, but neither method allowed for the analysis of DNA extracted from paraffin tumor sections. We are currently using the high-throughput denaturing high-performance liquid chromatography (DHPLC) assay coupled with dideoxy-sequencing and it is proving to be robust. We expect to have the mutational spectra from all 150 samples as well as the statistical analysis of the new data set complete by the end of this year.

BODY

4-ABP-DNA adducts

The immunohistochemical analysis of 4-ABP-DNA adducts in breast tumor tissue was completed in the first year of funding. Statistical analysis included determining the differences of adduct levels between smokers and nonsmokers. Associations were also determined between 4-ABP-DNA adduct levels and the smoking status variables; pack-years, age at initiation, and smoking intensity. In the second year of funding we used the same stained tumor sections to quantitate the 4-ABP-DNA adducts in the normal adjacent tissue. The data in Table 1 reveal that no significant associations were found between 4-ABP-DNA adduct levels and smoking exposure variables. This was observed in both breast tumor and normal adjacent tissue.

Genotyping

4-ABP is a potent human liver and bladder carcinogen and one of the major arylamines found in cigarette smoke. 4-ABP is not carcinogenic in the parent form but requires metabolic activation to reactive electrophiles in order to produce its carcinogenic effects. The grant proposed to determine polymorphisms in the enzymes responsible for metabolizing 4-ABP including CYP1A2 and NAT1. Recent publications found associations between NAT2 and GSTs and we included these genes in our genotypic analysis. The metabolizing enzymes possess a characteristic pattern of interindividual variation in human populations which may be determined by genotyping for single nucleotide polymorphisms.

In the second year of funding a high throughput genotyping method using the Fluorescence Polarization-dye-terminator incorporation assay was attempted. Although it worked well on DNA isolated from blood, the poor quality of DNA isolated from paraffin sections precluded its use. Analysis of the single nucleotide polymorphisms in CYP1A1, CYP1A2, NAT2, NAT1 and GSTP1 were carried out by the conventional PCR-RFLP assay. The statistical analysis revealed no significant associations between genotype and 4-ABP-DNA adduct levels in both tumor and normal adjacent tissues. Please see Tables 2, 3 & 4 in the Appendix.

P53 Gene Mutational Spectra

The proposed aim was to determine the mutations of the p53 gene in all 150 breast tumor samples and to make correlations between identified mutations and 4-ABP-DNA adduct levels. The original proposal planned to use the conventional SSCP method using large vertical polyacrylamide gels. Difficulties arose in obtaining a difference in band migration between the positive and negative controls. This time-consuming and obsolete method was deserted for the more efficient and comprehensive GeneChip method once we obtained additional funding with a pilot grant from the Women-at-Risk Foundation. This GeneChip had been validated using DNA extracted from frozen tumor section but we failed to validate this assay for DNA extracted from paraffin-embedded tumor sections. During the third and final year of funding we analyzed 130 samples hoping to identify which GeneChip mutation scores were noise or true mutations. Dideoxysequencing failed to confirm those mutations identified by the GeneChip.

We are currently using the DHPLC method to identify p53 mutations. We have a DHPLC instrument available to us in Columbia University's Genome Center. This high-throughput and inexpensive method, coupled with sequencing has proven to be sensitive and specific enough for our DNA samples. To date we have verified the mutations identified in exon 5 by DHPLC with sequencing. We anticipate that the remaining exons 7, 6, and 8 will be complete by the end of this month.

KEY RESEARCH ACCOMPLISHMENTS

- Determined that the 4-ABP-DNA adduct levels in normal adjacent breast tissue is slightly elevated, though not statistically significant, in nonsmokers compared to smokers.
- Determined the 4-ABP-DNA adduct levels in tumor tissue were slightly elevated, though modest and not statistically significant, in smokers with higher exposure in relation to pack-years.
- There were no statistically significant differences in 4-ABP-DNA adduct levels in either tumor or normal adjacent tissue between smokers stratified by age at initiation.
- The relationship between the genotypes for the carcinogen metabolizing enzymes, CYP1A2, NAT2, NAT1 and GSTM1 have in predicting 4-ABP-DNA adduct levels was determined. None of these genes, independently or in combination, had a significant association with adduct levels.
- The analysis of the mutational spectra of p53 is currently under investigation utilizing the DHPLC assay.
- Advanced coursework in epidemiology, statistics, toxicology and environmental health were completed during the timeframe of this project. The student also attended and gave presentations in the department's weekly seminar series.

REPORTABLE OUTCOMES

- American Association of Cancer Research Annual Meeting 2001 Abstract #3566 "4-Aminobiphenyl-DNA Adducts in Breast Tumor Tissue"

- Abstract for Era of Hope Meeting 2002 Poster Session Epidemiology II, P35-27 "4-Aminobiphenyl-DNA Adduct Damage in Breast Tissue and the Relationship to Polymorhpisms of Metabolizing Genes"

- We hope to publish this data in a medical journal with an emphasis on molecular epidemiology.

- The experience gained and data procured while completing this project will augment the requirements for the doctoral program at Columbia University Mailman School of Public Health.

CONCLUSIONS

The immunohistochemical analysis of breast tumor sections revealed that the difference in relative staining intensity for 4-ABP-DNA adducts was not significantly different between smokers, ex-smokers, and non-smokers. 4-ABP-DNA adduct levels were not significantly elevated in smokers with higher compared to lower exposure in relation to age at initiation or pack-years. These findings were observed in both breast tumor tissue and normal adjacent tissue. The polymorphisms in the enzymes that metabolize 4-ABP; CYP1A2, NAT2, NAT1, and GSTP1 were not associated to 4-ABP-DNA adducts in breast tumor tissue and normal adjacent. None of these genes, independently or in combination had a significant association with adduct levels. This data suggests that exposure to cigarette smoke does have an affect on breast cancer risk.

The lack of significant associations between 4-ABP-DNA adducts in breast tissue and smoking variables suggest that breast tissue may not be an appropriate medium for which to measure exposure to cigarette smoke. Passive smoking exposure data was not available in the parent study possibly causing a misclassification of nonsmokers and contributing to the lack of associations. It has been previously reported that sidestream smoke contains 30 times more 4-ABP than mainstream smoke strengthening the nonsmoker misclassification. Finally, this particular population is young and the inheritance of mutations in high-risk genes, such as BRCA1 and BRCA2, may play a larger role in breast cancer development rather than environmental exposures.

The lack of significant findings for the relationship between metabolizing enzymes and 4-ABP-DNA adduct levels in breast tissue may be due to the lack of information we have of the toxicokinetics of 4-ABP exposure in the breast. Experimental studies have shown that CYP1A2 is not the sole P450 enzyme responsible for metabolizing 4-ABP. *In vitro* metabolite studies show that another unidentified P450 is responsible for half of 4-ABP hydroxylation. Also, it is too early to know if the polymorphisms identified for this project may reveal true phenotypic differences in CYP1A2 activity. The CYP1A2 gene is highly inducible and this may override any genotypic variance in activity. At this time, breast cancer risk population studies that examined the relationship between NAT2 and cigarette smoking have been inconclusive. We may find that complex gene-gene interactions also contribute to the variable susceptibility of risk for breast cancer associated to cigarette smoking exposure.

The future analysis of this population will be to complete the p53 mutational spectra of all 150 samples for exons 5-8. This data will be examined to determine if a relationship exists between p53 mutations, 4-ABP-DNA adduct levels, and smoking variables in both breast tumor tissue and the normal adjacent. We will also correlate the mutation data to PAH-DNA adduct and p53 immunohistochemical staining previously determined in the same population. The results from this project have enhanced our understanding of the mechanisms responsible for the initiation and progression stages of chemical carcinogenesis. The correlations made with the p53 mutation data might help determine risk factors for breast cancer and possibly lead to modes of prevent

APPENDIX A

Table 1
Mean Relative Staining Intensities of 4-ABP-DNA adducts in Breast Tissue

	Tumor Tissue Mean (SD)	n	Normal Tissue Mean (SD)	n
Non-smoker	0.29 (.15)	73	0.32 (.16)	25
Ex-smoker	0.28 (.16)	40	0.30 (.10)	10
Smoker	0.31 (.17) *	36	0.24 (.09) **	11
Pack-years				
Nonsmoker	0.29 (.15)	73	0.32 (.16)	25
0 - <5	0.29 (.14)	29	0.32 (.10)	6
5 - <16	0.24 (.14)	20	0.20 (.05)	4
16+	0.33 (.19)**	27	0.26 (.09)**	11
Age at Initiation				
<16	0.29 (.22)	14	0.23 (.07)	6
16 – 18	0.33 (.16)	26	0.29 (.11)	6
18+	0.27 (.14)***	36	0.28 (.10)***	9
		*P > 0.5	**P = 0.3	***P =0.5

Table 2
Genotype Frequency

CYP1A2*1F	Wild	Heterozygous	Variant	n
NJ	42	46	12	151
NAT2		**Fast**	**Slow**	**n**
NJ		45	55	130

Table 3
Genotype and 4-ABP-DNA Adducts in Breast Tumor Tissue

CYP1A2*1F	relative stain (SD)		combined Wild+Heterozygous	
Wild	0.29(.14)		0.30 (.16)	
Heterozygous	0.31(.17)			
Variant	0.27(.14)	p=0.33	0.27 (.14)	p=0.16
NAT2				
Fast	0.31 (.16)			
Slow	0.27 (.15)	p=0.23		
NAT1				
Wild	0.28(.12)		0.28 (.14)	
Hetero	0.28(.15)			
Mutant	0.32(.18)	p=0.36	0.32 (.18)	p=0.15
GSTP1				
Wild	0.25(.14)		0.26(.15)	
Hetero	0.31(.15)			
Mutant	0.30(.15)	p=0.24	0.30(.15)	p=0.10

Table 4
Genotype and 4-ABP-DNA adducts in Breast Normal Tissue

CYP1A2*1F	relative stain (SD)		combined Wild+Heterozygous	
Wild	0.25 (.10)		0.30 (.11)	
Hetero	0.31 (.11)			
Mutant	0.29 (.16)	p=0.47	0.29 (.16)	p=0.86
NAT2				
Fast	0.33 (.12)			
Slow	0.28 (.15)	p=0.30		
GSTP1				
Wild	0.29 (.11)		0.30 (.15)	
Hetero	0.31 (.18)			
Mutant	0.30 (.13)	p=0.95	0.30 (.13)	p=0.89
NAT1				
Wild	0.25(.10)		0.28 (.13)	
Hetero	0.28(.14)			
Mutant	0.29(.10)	p=0.73	0.29 (.10)	p=0.79

Table 5

Breast Tumor Tissue	Mean relative staining for 4-ABP-DNA adducts (SD)		
	NAT2		
CYP1A2*1F	**fast**	**slow**	
(CC)	0.32 (.17)	0.29 (.17)	
(AA)	0.28 (.15)	0.24 (.12)	p=.21

Normal Breast Tissue	Mean relative staining for 4-ABP-DNA adducts (SD)		
	NAT2		
CYP1A2*1F	**fast**	**slow**	
(CC)	0.33 (.13)	0.29 (.12)	
(AA)	0.32 (.11)	0.28 (.18)	p=.79

APPENDIX B

American Association for Cancer Research
Annual Meeting, New Orleans April 2001
Abstract #3566

Title: 4-Aminobiphenyl-DNA Adducts in Breast Tumor Tissue

Authors: N Niguidula. Santella RM. Gammon MD. Terry MB. Schoenberg JB. Hibshoosh H.

Only a few epidemiological studies have shown a slight increased risk of breast cancer in cigarette smokers. The risk is only seen when variables of smoking status or genotypic variance in metabolizing enzymes are considered. To evaluate DNA damage due to smoking exposure we studied 4-aminobiphenyl(4-ABP)-DNA adduct levels in breast tumor tissue in a population-based study of 150 cases from New Jersey. All subjects were under the age of 45 with either *in situ* or invasive breast cancer. Adducts in breast tumor sections of paraffin blocks were measured utilizing an immunohistochemical peroxidase assay. The difference in relative staining intensity for 4-ABP-DNA adducts was not significantly different between smokers 0.32 (0.18), ex-smokers 0.29 (0.16), and non-smokers 0.29 (0.15). 4-ABP-DNA adduct levels was not significantly elevated in smokers with higher compared to lower exposure in relation to age at initiation or pack-years. This data suggests that exposure to cigarette smoke does have an affect on breast cancer risk. An increased risk of breast cancer has been found when taking into account smoking status as well as polymorhpisms related to variation in metabolism of carcinogens. We are currently evaluating single nucleotide polymorphisms in cytochrome P450 (CYP1A2), N-acetyltransferase (NAT1 and NAT2), and glutathione S-transferase (GSTP1) utilizing the highly specific and sensitive fluorescence polarization method. We will determine the relationship between adduct levels, genotypic variance, and the effect of smoking.

Era of Hope Meeting, Orlando, Florida, September 27, 2002
Poster Session Epidemiology II, P35-27

"4-AMINOBIPHENYL-DNA ADDUCT DAMAGE IN BREAST TISSUE AND THE RELATIONSHIP TO POLYMORPHISMS OF METABOLIZING GENES"

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Only a few epidemiological studies have shown a slight increased risk of breast cancer in cigarette smokers. The risk is only seen when variables of smoking status or genotypic variance in metabolizing enzymes are considered. To evaluate DNA damage due to smoking exposure we studied 4-aminobiphenyl(4-ABP)-DNA adduct levels in breast tumor tissue in a population-based study of 150 cases from New Jersey. All subjects were under the age of 45 with either *in situ* or invasive breast cancer. Adducts in breast tumor sections of paraffin blocks were measured using an immunohistochemical peroxidase assay. The difference in relative staining intensity for 4-ABP-DNA adducts in tumor tissue was not significantly different between smokers 0.31 (0.17), ex-smokers 0.28 (0.16), and non-smokers 0.29 (0.15). 4-ABP-DNA adduct levels were not significantly elevated in smokers with higher compared to lower exposure in relation to age at initiation or pack-years. This data suggests that exposure to cigarette smoke might have an affect on breast cancer risk.

4-ABP is not carcinogenic in the parent form but requires metabolic activation to reactive electrophiles in order to produce its carcinogenic effects. An increased risk of breast cancer has previously been found when smoking status as well as polymorphisms related to variation in metabolism of carcinogens was evaluated. We evaluated single nucleotide polymorphisms in the enzymes that have been found to metabolize 4-ABP; cytochrome P4501A2 (CYP1A2), two N-acetyltransferases, NAT1 and NAT2, and glutathione-S-transferase(GST). None of these genes, independently or in combination, had a significant association with adduct levels. Passive smoking exposure data was not available in the parent study possibly causing a misclassification of nonsmokers and contributing to the lack of associations. Breast tissue may not be an appropriate medium to measure damage from cigarette smoking exposure. Finally, this particular population is young and the inheritance of mutations in high-risk genes, such as BRCA1 and BRCA2, may play a larger role in breast cancer development rather than environmental exposures.

The conclusions drawn from this project have shown that cigarette smoking may have an affect on breast cancer risk and DNA damage levels in breast tissue is not modified by individual susceptibility markers of carcinogen metabolism.

The U.S. Army Medical Research and Materiel Command under DAMD17-99-1-9155 supported this work.